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Research Article

Differential response between the p53 ubiquitin–protein ligases Pirh2 and MdM2 following DNA damage in human cancer cells

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ABSTRACT

Pirh2, a recently identified ubiquitin–protein ligase, has been reported to promote p53 degradation. Pirh2 physically interacts with p53 and promotes ubiquitination of p53 independently of MDM2. Like MDM2, Pirh2 is thought to participate in an autoregulatory feedback loop that controls p53 function. We have previously reported that Pirh2 was overexpressed in human and murine lung cancers as compared to uninvolved lung tissue. Pirh2 increase could potentially cause degradation of wildtype p53 and reduce its tumor suppression function in the lung tumor cells. Since Pirh2 has been reported to be transactivated by p53, however, the mechanisms by which a high level of Pirh2 expression is maintained in tumor cells despite low level of wildtype p53 protein are unclear. In order to evaluate p53 involvement in the transactivation of Pirh2, we evaluated Pirh2, MDM2, p53 and p21 expression with Western blot analysis and real time PCR after γ irradiation or cisplatin DNA damage treatment using human cancer cell lines containing wildtype (A549, MCF-7), mutant (H719) and null (H1299) p53. Surprisingly, Pirh2 expression was not affected by the presence of wildtype p53 in the cancer cells. In contrast, MDM2 was upregulated by wildtype p53 in A549 and MCF-7 cells and was absent from the H1299 and the H719 cells. We conclude that Pirh2 operates in a distinct manner from MDM2 in response to DNA damage in cancer cells. Pirh2 elevation in p53 null cells indicates the existence of additional molecular mechanisms for Pirh2 upregulation and suggests that p53 is not the sole target of Pirh2 ubiquitin ligase activity.

Introduction

The tumor suppressor protein p53 is a key regulator of cell cycle control, apoptosis and genomic stability in response to various cellular stresses [1–6]. Mutation of the p53 gene is the most frequently reported genetic defect in human cancers [7,8]. The best characterized and most important function of wildtype p53 is the sequence specific transactivation of target genes [1,9]. In normal cells, the steady state level of p53 protein is very low. However, DNA damage induces a prominent

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increase in p53. The increased wildtype p53 protein transactivates expression of p21, bax and other important genes that are involved in cell cycle control, apoptosis and genomic stability [2–4,10,11]. The genomic guardian function of p53 is exerted through either cell cycle arrest or apoptotic cell death [12]. Loss of wildtype p53 not only disrupts cell cycle checkpoints and apoptosis, but also results in centrosome hyperamplification that leads to defective mitosis organized by multiple spindle poles [13]. The p53 null ($p53^{-/-}$) mice are developmentally normal but highly susceptible to the early development of spontaneous tumors [14,15].

MDM2 was initially identified as an amplified oncogene in murine cell lines [16] and in sarcomas [17] and was subsequently found to act as a ubiquitin ligase promoting proteasome dependent degradation of p53 [18–20]. MDM2 is also a transcriptional target of p53 [21–23], such that p53 activity controls the expression and protein level of its own negative regulator, providing for an elegant feedback loop. MDM2 inhibits the G1 arrest and apoptosis functions of the p53 tumor suppressor protein [24,25]. The MDM2–p53 complex also inhibits p53 mediated transactivation [26].

Pirh2, a recently reported ubiquitin–protein ligase, has been reported to promote p53 degradation [27]. The Pirh2 gene encodes a RING-H2 domain-containing protein with intrinsic ubiquitin–protein ligase activity. Pirh2 physically interacts with p53 and promotes ubiquitination of p53 independently of Mdm2. Pirh2 was also reported to be transactivated by the p53 product in MEFs, murine proB cell BaF3 and human BJT fibroblasts cells. Therefore, like MDM2, Pirh2 has been thought to participate in an autoregulatory feedback loop that controls p53 function [27]. The wildtype Pirh2 protein is unstable and has a short half-life. Coexpression of TIP60 (Tat-interactive protein of 60 kDa) enhanced Pirh2 protein stability and altered Pirh2 subcellular localization [28].

In a previous report, we found that Pirh2 was overexpressed in both human and murine lung cancers by comparing Pirh2 mRNA and protein level between lung neoplastic tissues and uninvolved adjacent lung tissue [29]. The increased Pirh2 protein could cause degradation of wildtype p53 and reduce the tumor suppression function in the lung tumor cells. However, cancer cells maintained a high level of Pirh2 protein despite low levels of p53 protein, a characteristic not generally observed in the MDM2–p53 autoregulatory feedback loop. To obtain a better insight in the behavior of Pirh2 as it relates to p53, we evaluated Pirh2, MDM2, p53 and p21 expression using cell lines containing wildtype, mutant and null p53 after DNA damage treatment.

Methods

Cell culture, plasmids and reagent

Human lung cancer cell lines A549 (wildtype p53); H23 (mutant p53, residue 246 from M to I); H125 (p53 frame shift at codon 239 and protein truncated at residue 245); H211 (mutant p53, residue 248, from R to Q); H719 (mutant p53, residue 242 from C to W); H1155 (mutant p53, residue 273 from R to H); and H1299 (p53 null) were obtained from the American Type Culture Collection (ATCC, Rockville, MD) and were grown in RPMI 1640 medium with 10% of fetal bovine serum and 1% penicillin/streptomycin in an incubator (37°C, 5% of CO_2). Human mammary epithelial MCF-7 cell (wildtype p53) and MEFs were grown in D-MEM medium with 10% of fetal bovine serum and 1% penicillin/streptomycin. A549 cells with the wildtype human papilloma virus type-16 E6 gene (HPV E6) (A549/E6w) cell line was generated by stably transfecting A549 cells with pCMV-neo-E6 plasmid and selected using 400 μg/ml G418 as described previously [30,31]. H1299 cells with wildtype p53 were generated by transfection of pCMV-p53w (human wildtype p53) using Transfection kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Human lung tumors and normal tissue samples were obtained from The Cooperative Human Tissue Network, MidWestern Division at The Ohio State University.

Real time PCR

Total RNA was isolated from the cell lines using Trizol RNA isolation following the protocol supplied by the manufacturer (GIBCO BRL, Rockville, MD). RNA samples were treated with DNase (Ambion Inc, Austin, TX) to remove contaminating DNA and stored in −70°C freezer.

Primers used for the real time PCR analysis were designed according to mRNA sequences (Genbank Accessions: NM_026557 for murine Pirh2, NM_010786 for murine MDM2, NM_015436 for human Pirh2 and BT007258 for human MDM2). Primers used for the real time PCR were hMDM2F120 (5′-TGTTGGTGCACAAAAAGACA) and hMDM2R264 (5′-CACGCCAAACAAATCTCCTA) for hMDM2; primers hPirh2F82 (5′-GCTGCGAGCACTATGACAGA) and hPirh2R213 (5′–TGCACTGCACTTCCT TCACT) for human Pirh2; primers mPirh2F102 (5′-GCTGCGAGCACTATGACAGA) and mPirh2R195 (5′-TGATCTTCATTGGTATCGTGACA) for murine Pirh2; and primers MDM2F1220 (5′-CTTCGTGAGAACTGGCTTCC) and MDM2R1346 (5′-CTGTCAGCTTTTTGCCATCA) for murine MDM2. Primers used to amplify ribosomal internal controls were obtained from Applied Biosystems (Applied Biosystems, Foster City, CA). Primers were tested with reverse transcription PCR (RT-PCR) amplification followed by an agarose gel electrophoresis to ensure the accuracy of the amplification. The RT-PCR amplification was conducted in 25 μl reaction using the Qiagen OneStep RT-PCR kit (Qiagen, Valencia, CA). Each reaction contained 400 μM of each dNTP, 1× RT-PCR buffer, 10 pmol of each primer, 1 μl OneStep RT-PCR Enzyme mixture and 200 ng of total RNA. Other conditions were performed as described previously [32].

The real time PCR amplification was conducted in a 25 μl reaction using the QuantiTect Sybr Green RT-PCR Kit (Qiagen, Valencia, CA) according to the protocol supplied by the manufacturer. 100 ng total RNA was used for each reaction. The real time PCR amplification conditions were 50°C for 30 min, 95°C for 15 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. Each sample was done in triplicate, and each reaction was repeated once to ensure accuracy. The PCR cycle number at threshold (CT) was used for the comparison. The relative quantitative method was used for the quantitative analysis. Calibrator was the averaged ΔCt from the untreated cells. Reactions were carried out in a 96-well plate